

Cellulect - A Synthetic Biology Platform for the Optimization of Enzymic Biomass Processing

Project acronym: CELLULECT

Project no: EIB.12.041

Prof. Alistair Elfick

ERA-IB-2 final conference, Berlin, 16./17.02.2016

Project partners

- *Co-ordinator*

Edinburgh, Scotland

- *Partners*

Centre de recherches interdisciplinaires

Paris, France

Marburg, Germany

ingenza

Edinburgh, Scotland

- *Total project budget:* €1.7M

Introduction

- *Project objective*

Meet the challenge of feedstock variability in biomass conversion by creating agile approaches to degradation tuning

Introduction

- *General project approach*

"Develop a technology pipeline applying novel rapid combinatorial genetic methods supported by bioinformatics, expression tuning and efficient screening of enzyme / accessory protein cocktails."

Technical overview



- *Work Package 1: Management*
- *Work Package 2: Enzyme ID & Capture*
- *Work Package 3: Expression Control Technologies*
- *Work Package 4: HTP Combinatorial Assembly*
- *Work Package 5: Performance Mapping*
- *Work Package 6: Numerical Modelling*

WP2: Enzyme Capture

Establish enzyme database → Select strains → Clone enzyme candidates into modular format → Assembly DNA parts for expression → Individual enzyme screening → Choose active candidates for next stage test → Tests of mixed enzyme with natural substrate → Test of mixed enzyme with industrial level biomass → Deliver the best recombination of biomass degradation

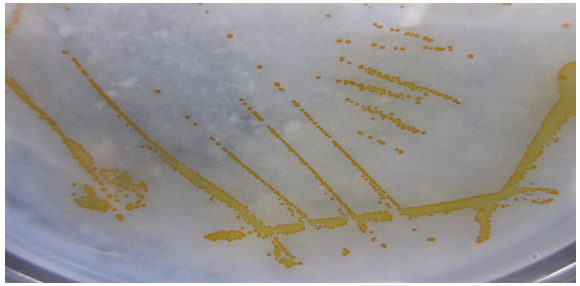
WP2: Database of Cellulase & Hemi

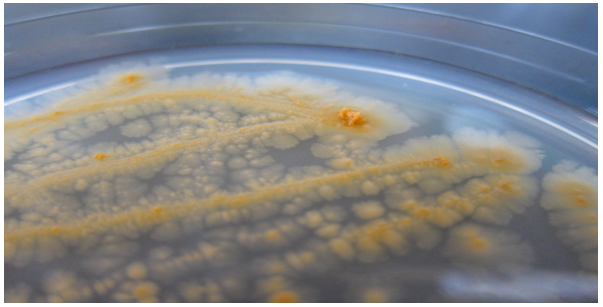


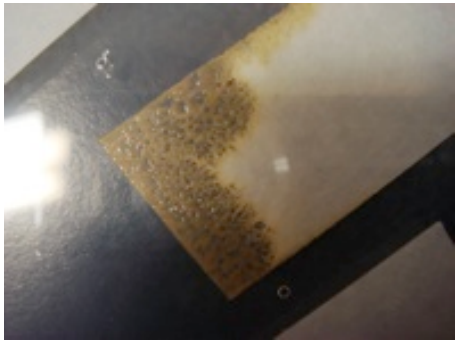
Biomining analysis:

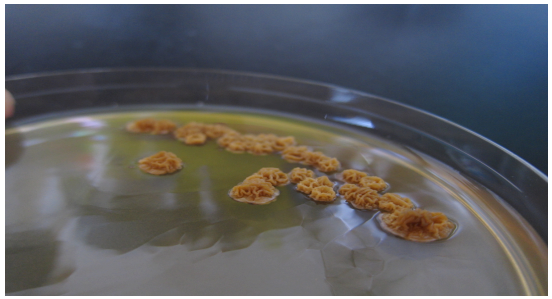
- More than 20,000 cellulases and hemicellulases were retrieved from UniProt (http://www.uniprot.org/)
  - including GH61 and CBM33 candidates
- 2,913 bacterial species and 327 fungal species with total 11,887 entries were found
- More than 95% of enzymes are annotated by Bioinformatics tools analysis

WP2: Strain selection

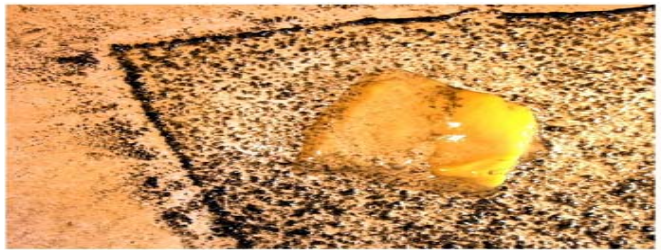








[Schneiker *et al*. NBt '07]



Actinoplanes missouriensis 431

Cellulomonas fimi ATCC 484

Cytophaga hutchinsonii ATCC 33406

Formosa agariphila KMM 3901

Micromonospora lupini Lupac 08

Sorangium cellulosum So ce 56

Teredinibacter turnerae T7901

WP2: Enzyme Capture

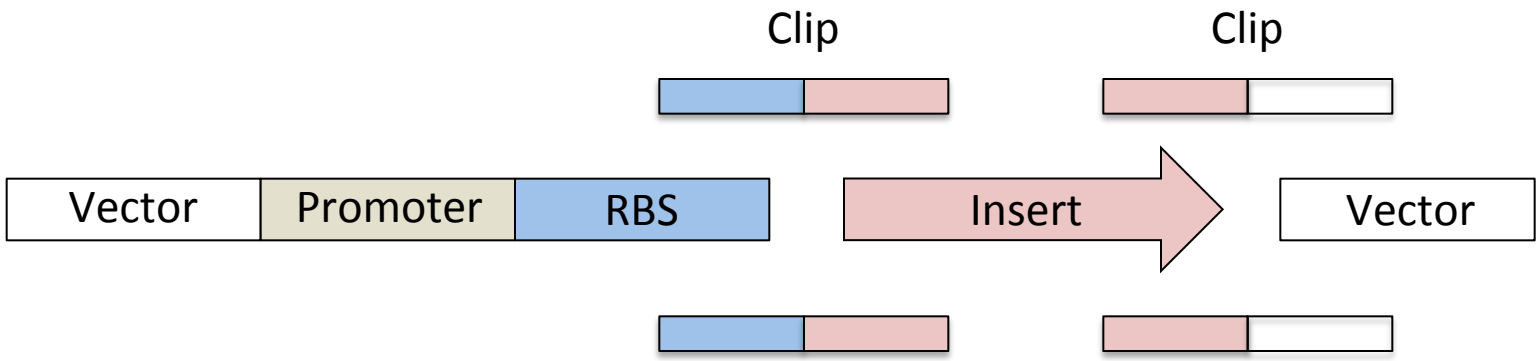


	Endoglucanase	Exoglucanase	b-Glucosidase
A. missouriensis 431	13	4	16
M. lupini lupac 08	6	2	11
S. cellulosum so ce56	10	2	9
T. turnerae T7901	17	2	4

Additionally, synthesized fungal GH61 sequences:

- *Podospora anserina*
  - Pa61A (Bey *et al*., 2013)
- *Neurospora crassa* OR74A
  - Nc61A (Sigmond *et al*., 2012)

WP2: Cloned into Modular Format



PaperClip: rapid multi-part DNA assembly from existing libraries

(Trubitsyna *et al*., '14)

Automated assembly

(de las Heras *et al*., in prep)

WP2: Individual Enzyme Screening



Each enzyme assayed individually for endocellulase, exocellulase and β-glucosidase activity

Assays:

4-Methylumbelliferyl ß-D-glucopyranoside (MUG)

4-Methylumbelliferyl ß-D-cellobioside (MUC)

4-Methylumbelliferyl ß-D-xylopyranoside (MUX)

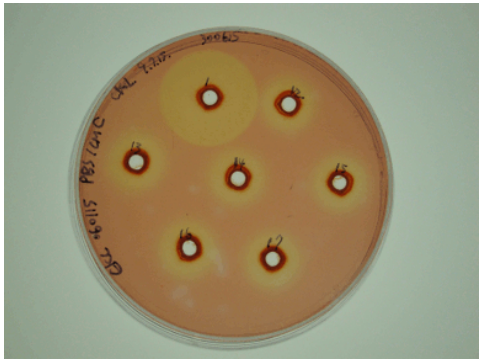
AZO-CMC

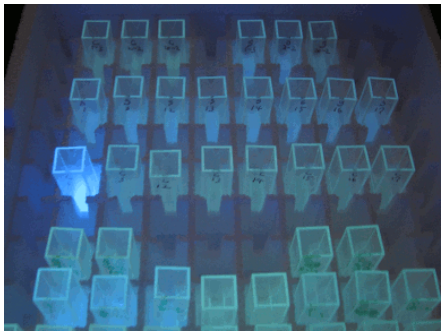
AZO-Xylan

Congo Red-CMC

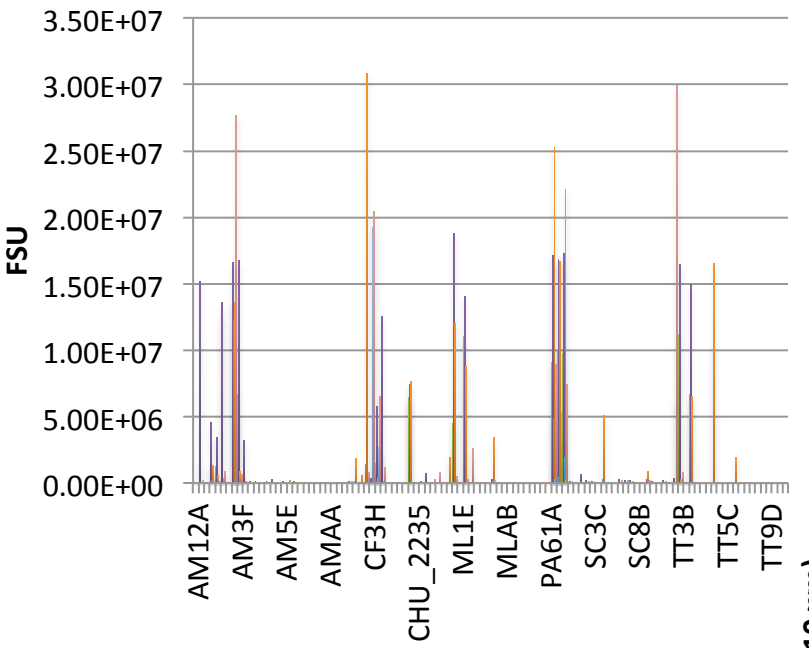
4-Nitrophenyl ß-D-mannopyranoside (PNPMan)

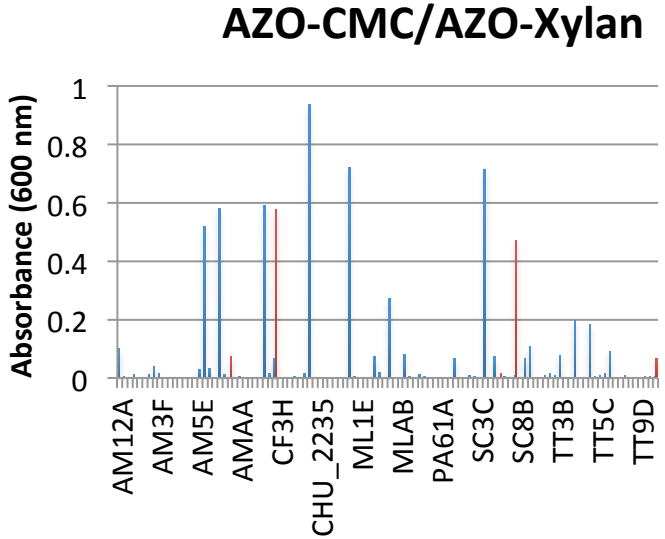
2-Nitrophenyl-ß-D-galactopyranoside (ONPG)

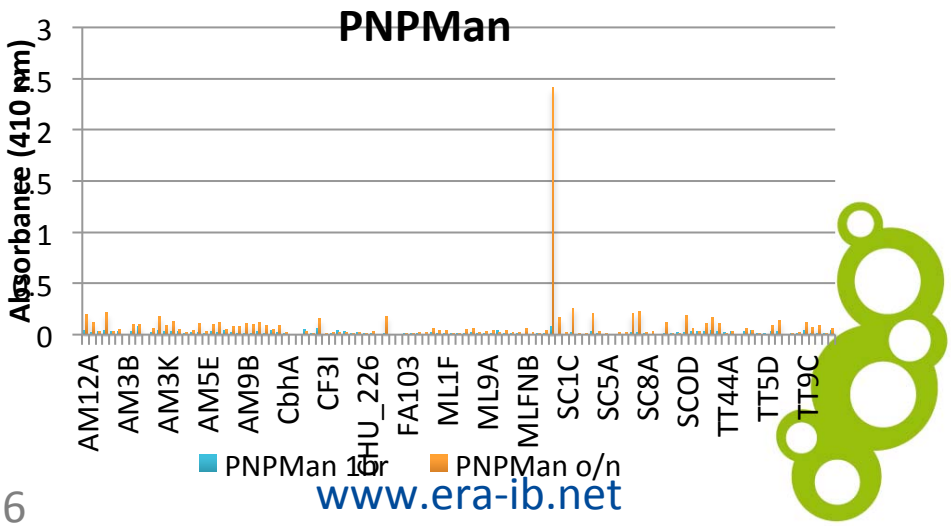




WP2: Individual Enzyme Screening







WP2: Candidates for Recombination

Endoglucanase	Exoglucanase	β-Glucosidase
AM12A	Cex	AM3D
AM5G	ML44A	CHU_2268
AM6B		ML1B
CenA		ML1F
CHU_2103		SC1A
ML12A		SC1B
ML5B		SC1C
SC5A		TT3D
SC5B		
TT44A		
TT5A		

- CBM33 and GH61 enzymes also tested
- 96-well plate reader will be used for recombination tests

WP2: Individual Enzyme Screening



Each enzyme assayed individually for endocellulase, exocellulase and β-glucosidase activity

Assays:

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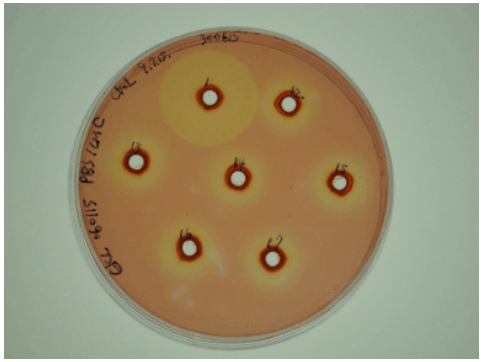
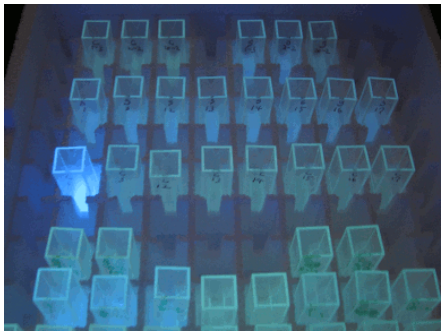
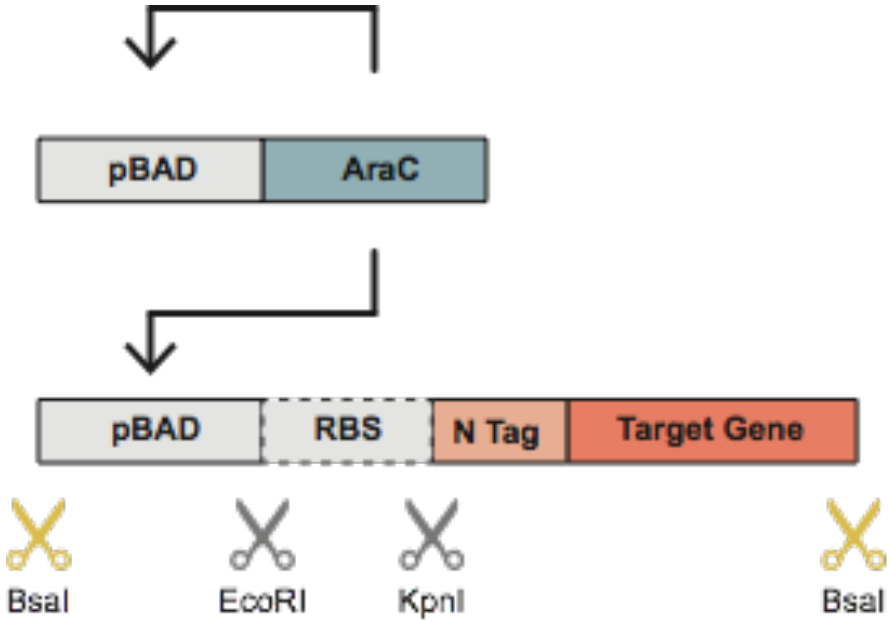




WP3: Expression Control Techs



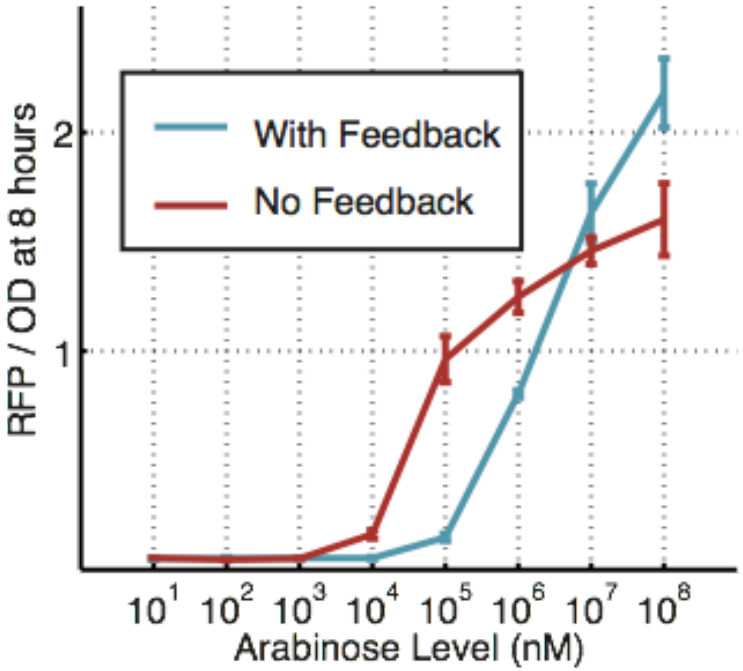
- Standard N-terminal tags
- Rationally designed RBS sequence
- Feedback-controlled inducer to smooth gene expression
- Standard BsaI sites for Golden Gate assembly

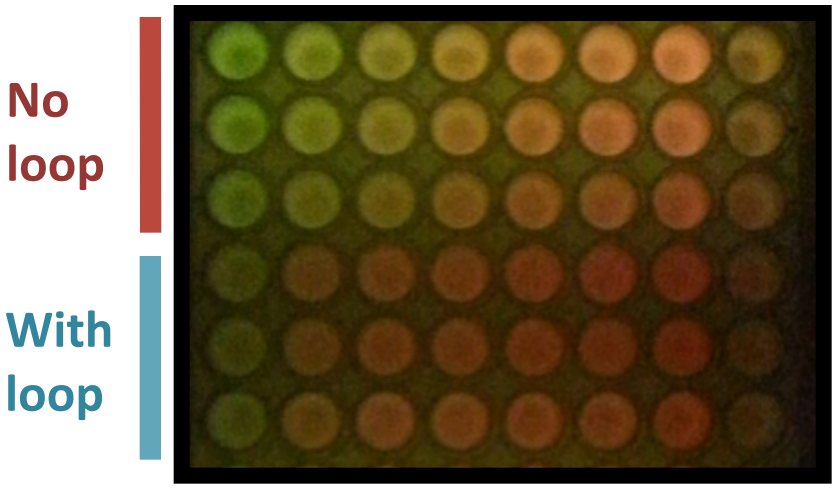


Construct Design: Linear, Modular, Standardized

WP3: Feedback smoothing

- Strains receive feedback loop or classical constitutively-expressed AraC inducer



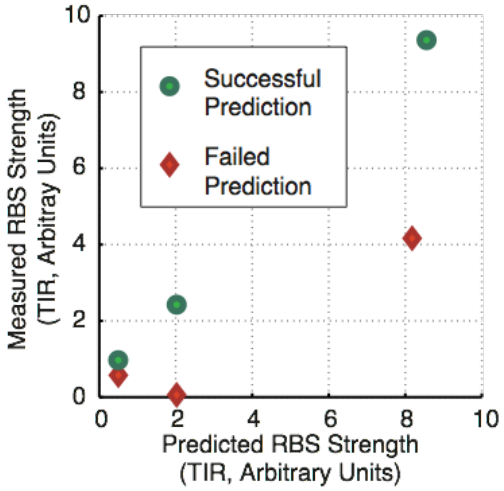


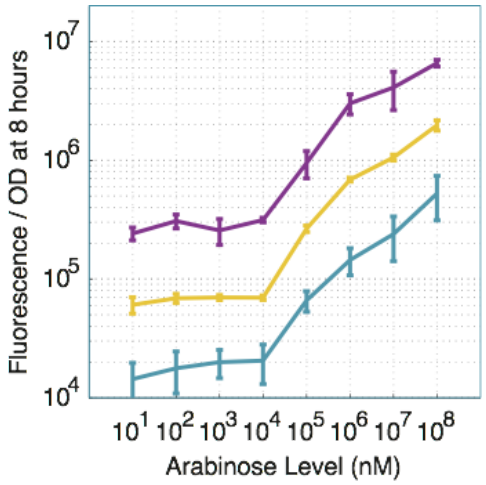
WP3: RBS optimisation



- Low, medium and high strength RBS sequences – extend the linear range of expression by >10 fold

Sequence	TIR
AATTCATTAAAG**ACTG** **A**GGAGGTAC	8555 3
AATTCATTAAAG**ATTA** **A**GGAGGTAC	8178 8
AATTCATTAAAG**AACG** **G**GGAGGTAC	2026 7
AATTCATTAAAG**CACA** **G**GGAGGTAC	2026 7

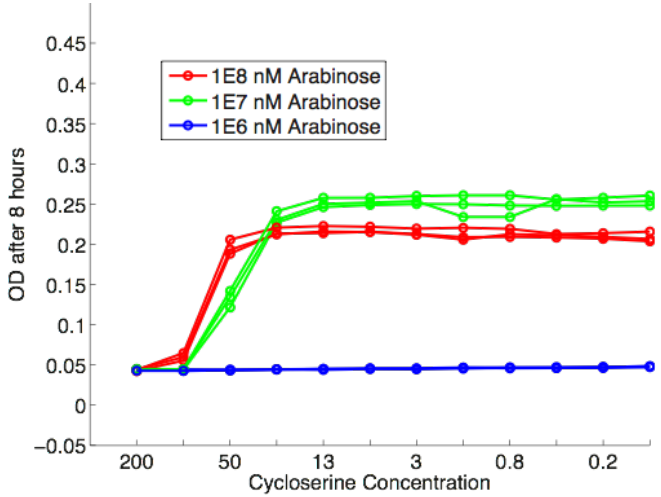




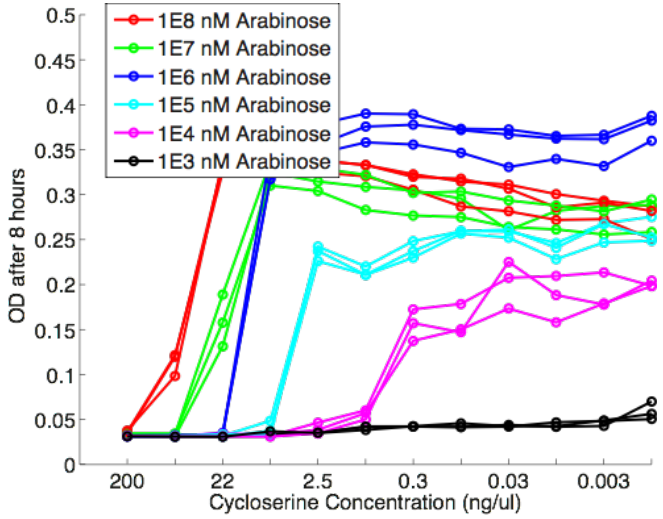
Generate RBS predictions with Salis Calculator → Verify RBS strength (50% success) → RBS effects are linear over the full range of induction

WP3: Test Case – Alanine racemase

- An essential metabolic pathway inhibited by cycloserine
- High expression is associated with 50% reduced growth
- Feedback control allows a much wider range of viable induction levels



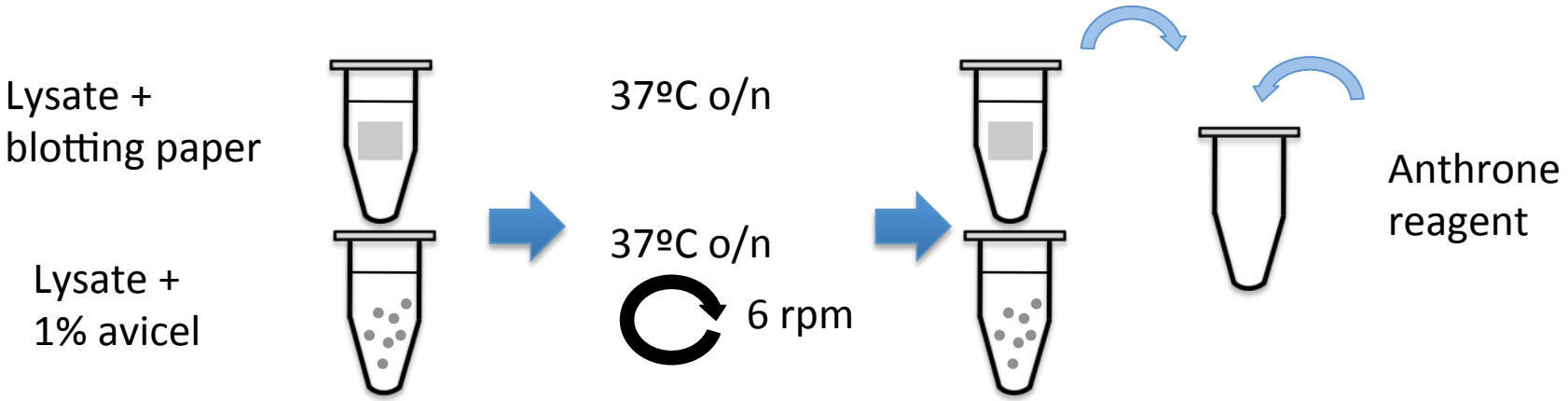
No Feedback

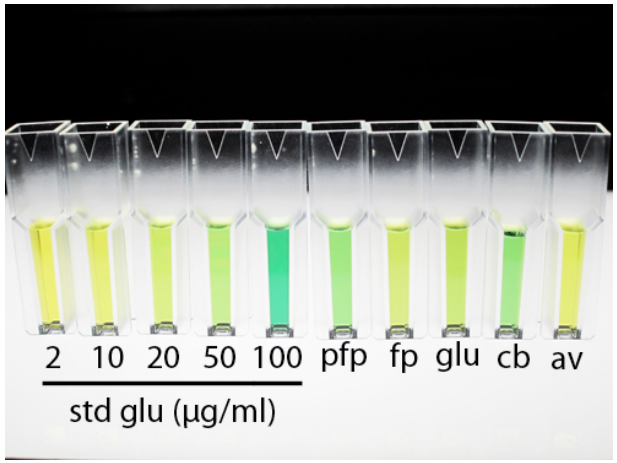


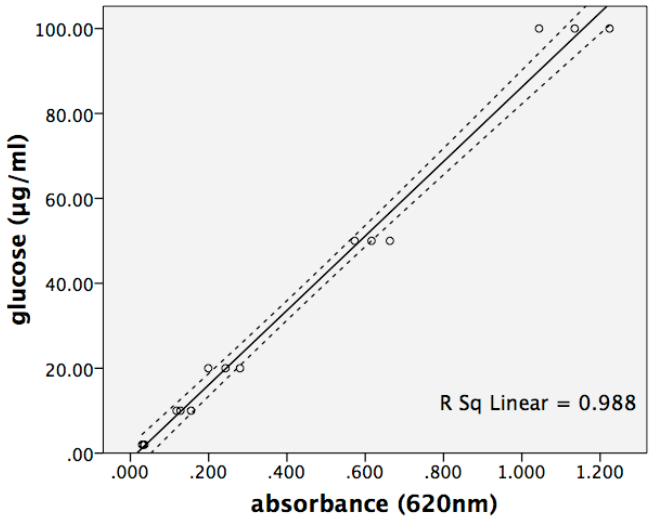
Feedback Control

WP4: Combinatorial Enzyme Assembly

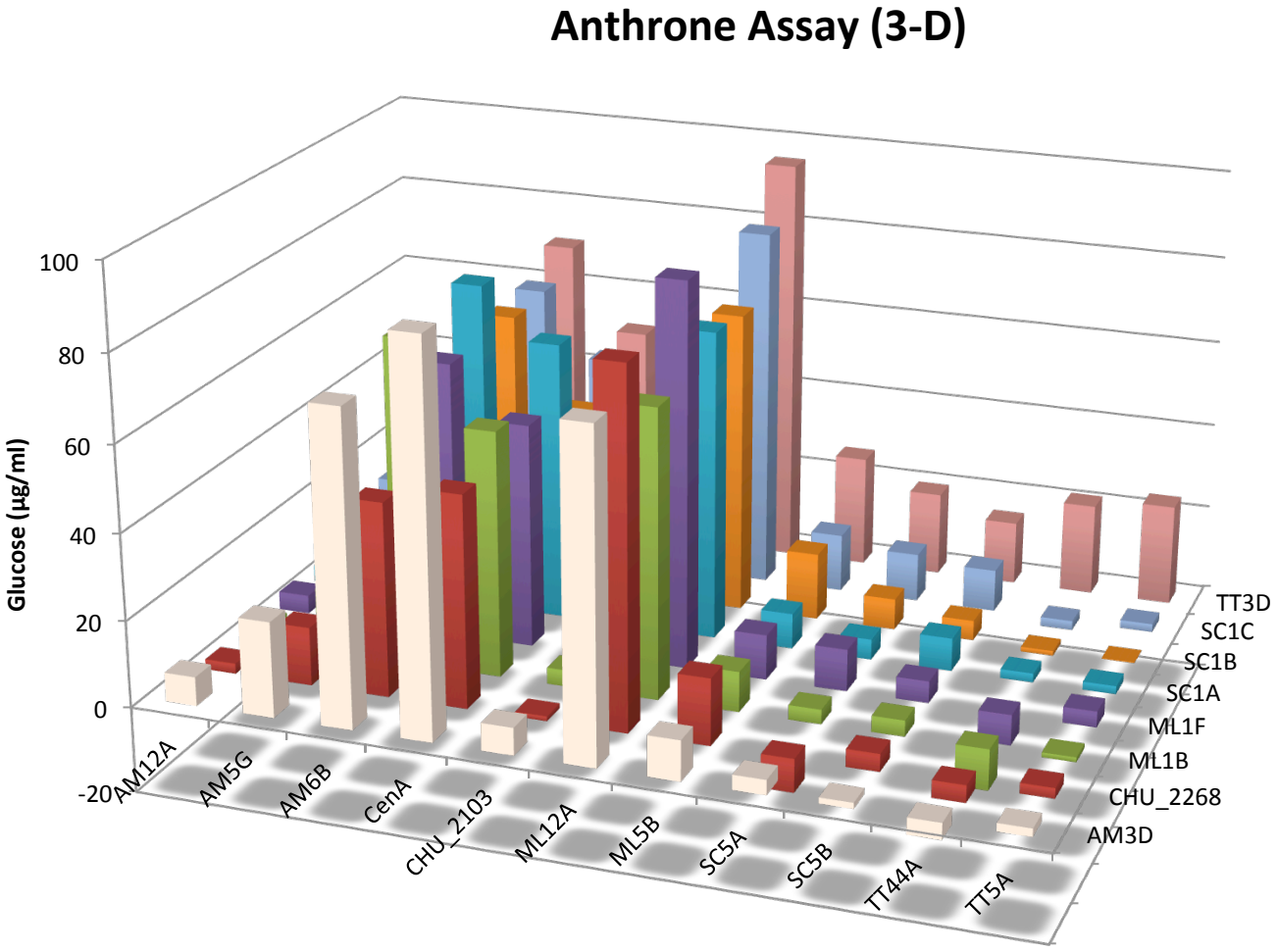








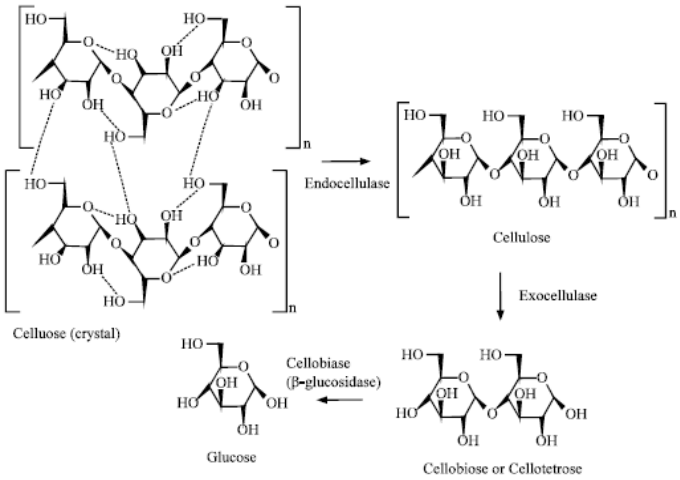
WP4: Combinatorial Enzyme Assembly



WP5: Enzyme ID & part design



Name	Organism	Characteristics
EX1	*Cytophaga*	May release glucose rather than cellobiose, potential β-glucosidase activity
EX2	*Cellulomonas*	Bi-functional xylanase-exoglucanase
EX3	*Cellulomonas*	EX2 fused with endoglucanase domain
EX4	*Cellulomonas*	Classical non-reducing-end cellobiohydrolase
EX5	*Cellulomonas*	Reducing-end-directed cellohiohydrolase

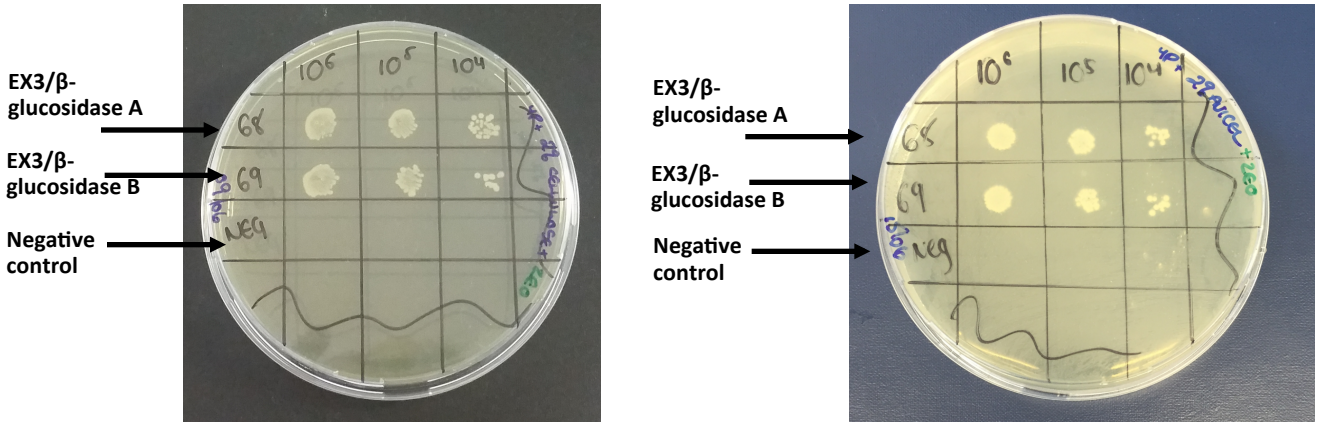


- WT sequences codon optimised for *S. cerevisiae*
- Truncated parts and oligos being used to construct expression cassettes
- EX1, EX2 and Ex3 focused on

WP5: Screening EX3/β-G constructs

Method

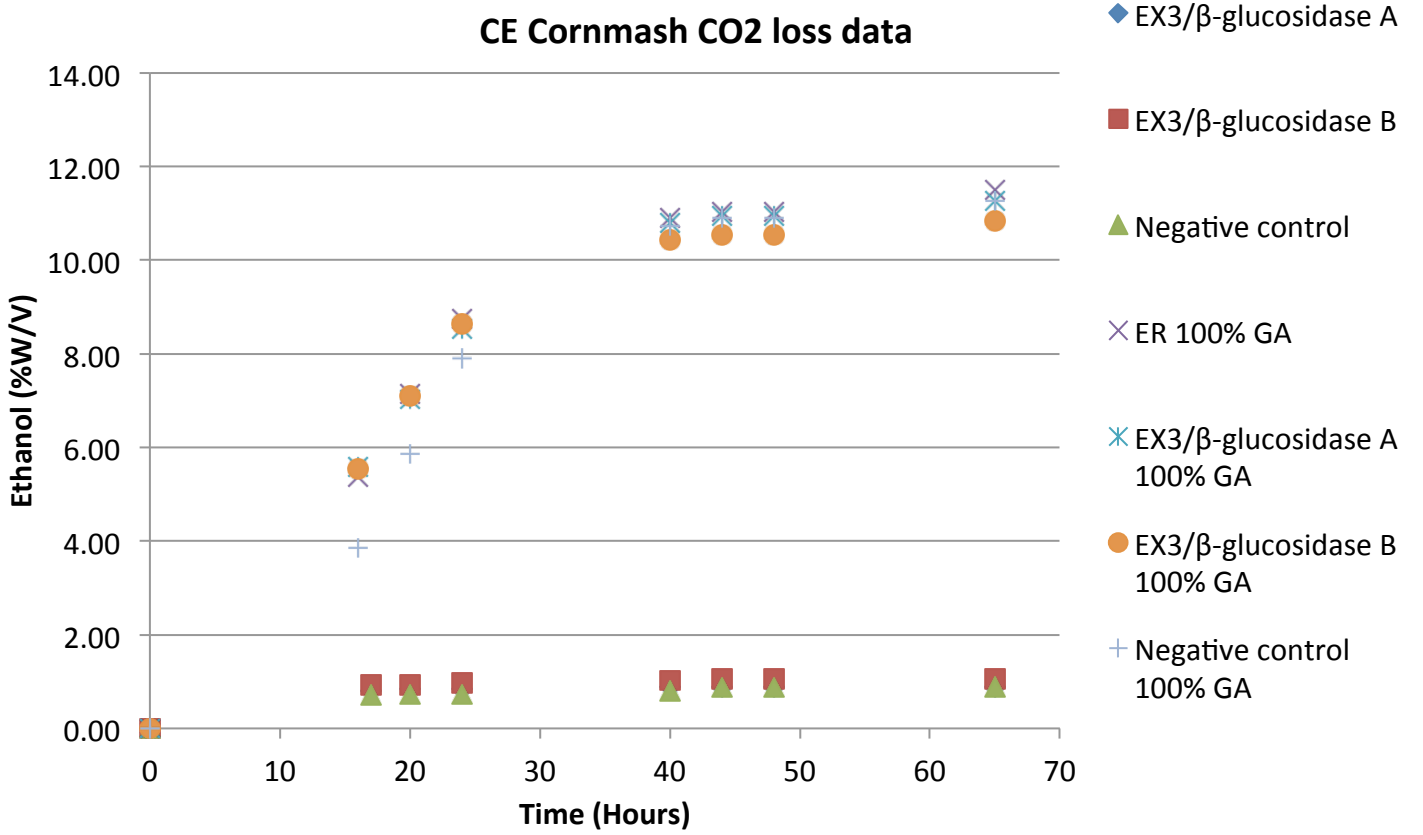
- Two EX3/β-glucosidase vectors and negative control assayed for growth on cellulose substrates.
- CM-Cellulose and Avicel used as substrates.
- Cells washed to remove residual glucose from culture media and spotted onto plates. Plates incubated for two days at 30 °C.



CM-Cellulose Avicel

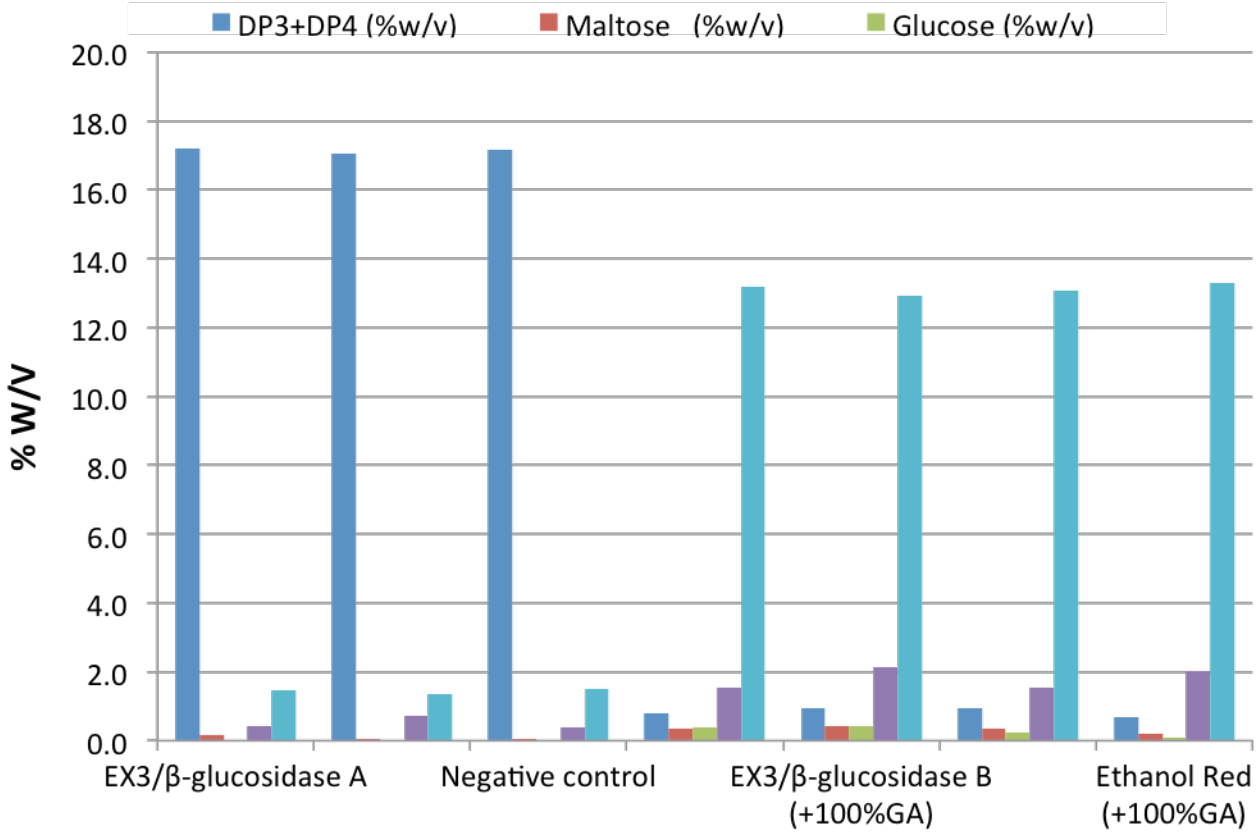
Since strains display the ability to grow on cellulose substrates they were assayed for the ability to access cellulose present in corn mash

WP5: Screening EX3/β-G constructs



Weight loss data highlights engineered strains producing ethanol at an increased rate than the control in the presence of 100% glucoamylase

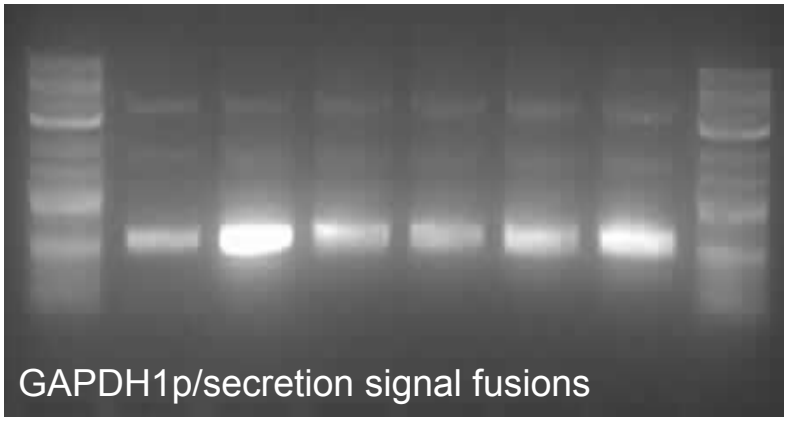
WP5: Screening EX3/β-G constructs

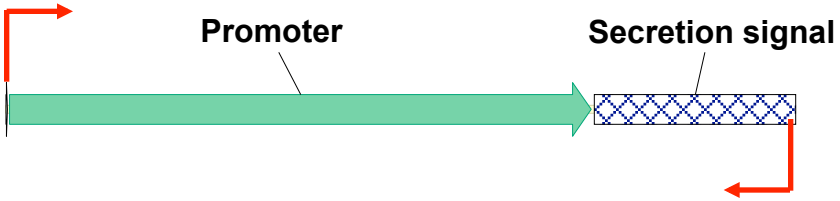


End-point HPLC analysis did not reveal a higher final ethanol

WP5: Promoter secretion signal library

- Promoter/secretion signal fusion PCR
- 8 secretion signal sequences identified.
- Four *S. cerevisiae* promoters selected from Ingenza inABLE database
- Primers designed and PCR's successfully performed to construct 32 promoter/secretion signal fusions





Promoter	Secretion signal
ADH1p	A. niger alpha Amylase
PGK1p	*A. awarmori* Glucoamylase
GAPDH1p	*H. sapien* serum albumin
PYF1p	*K. maxianus* Inulinase
	S. cerevisiae Invertase
	S. cerevisiae M1 killer toxin
	G. gallus Lysozyme
	S. cerevisiae α factor

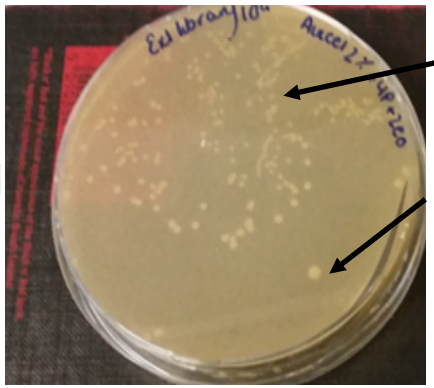
WP5: Promoter secretion signal library

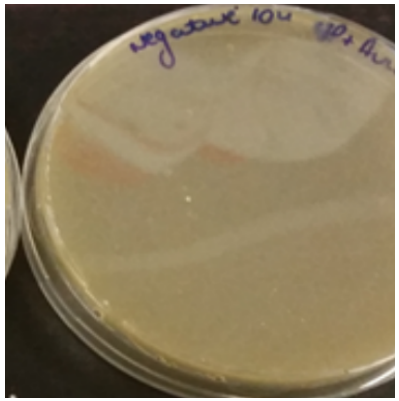
- Libraries constructed through three part assembly
- 32 combinations successfully isolated per library (EX1 and EX2)
- *S. cerevisiae* transformed and library currently screened on Avicel

EX1 library

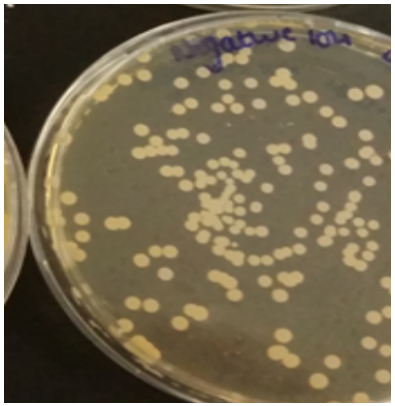
Negative Control

YP + 2% Avicel





YPD



WP6: Numerical Stochastic Model



- *Goal:*

Provide detailed description of concerted action of enzymes that transform cellulose into glucose

- *Ingredients:*

Substrate: cellulose is represented as 3-dimensional bundle of polymer chains

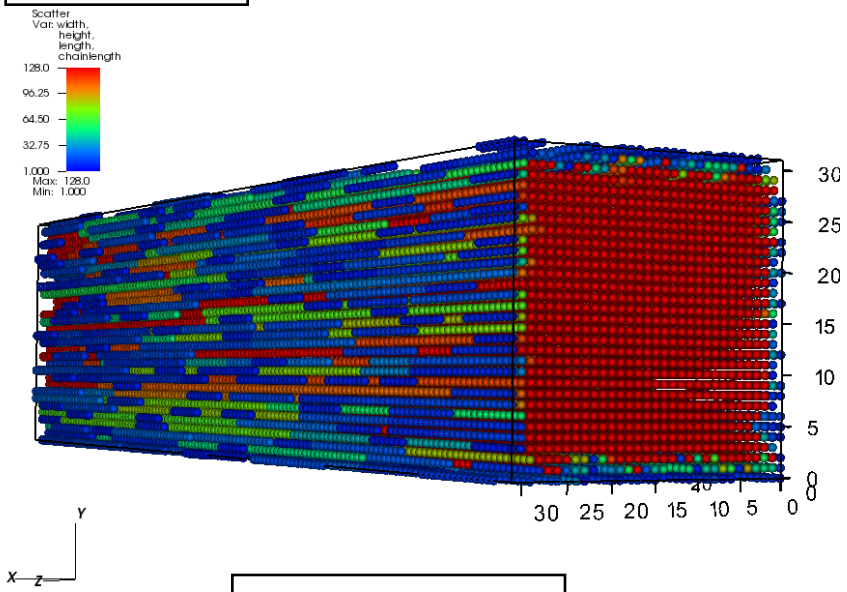
- *Applications:*

Find enzyme mixture (consisting e.g. of EG I or II, CBH I or II, βG etc.) that optimizes production of glucose

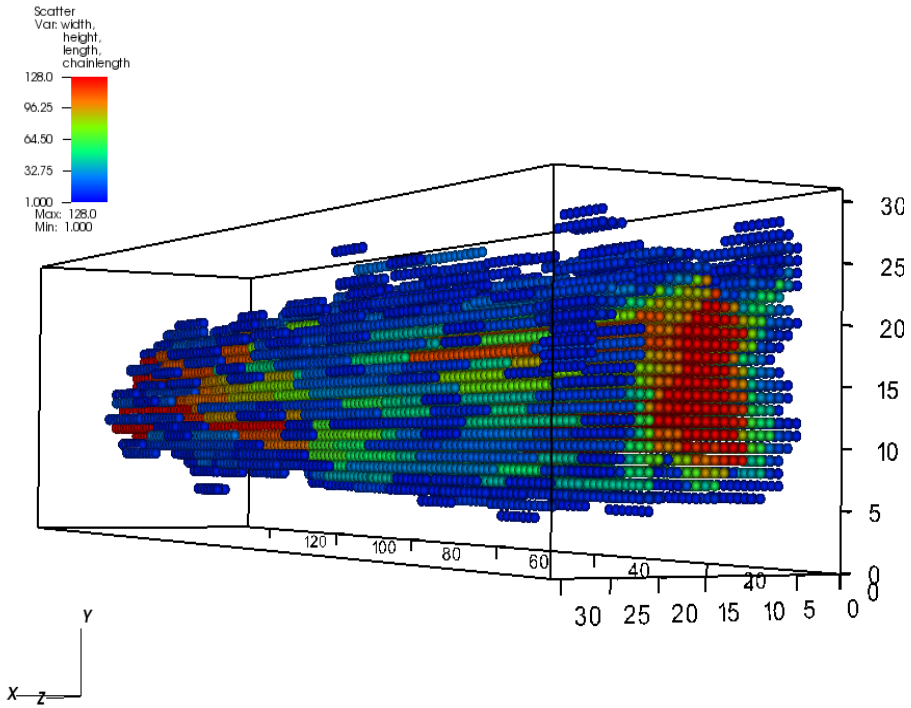
Analyse influence of substrate properties (geometry, crystallinity, degree of polymerization etc.) on this process

WP6: Substrate degradation

chain-length

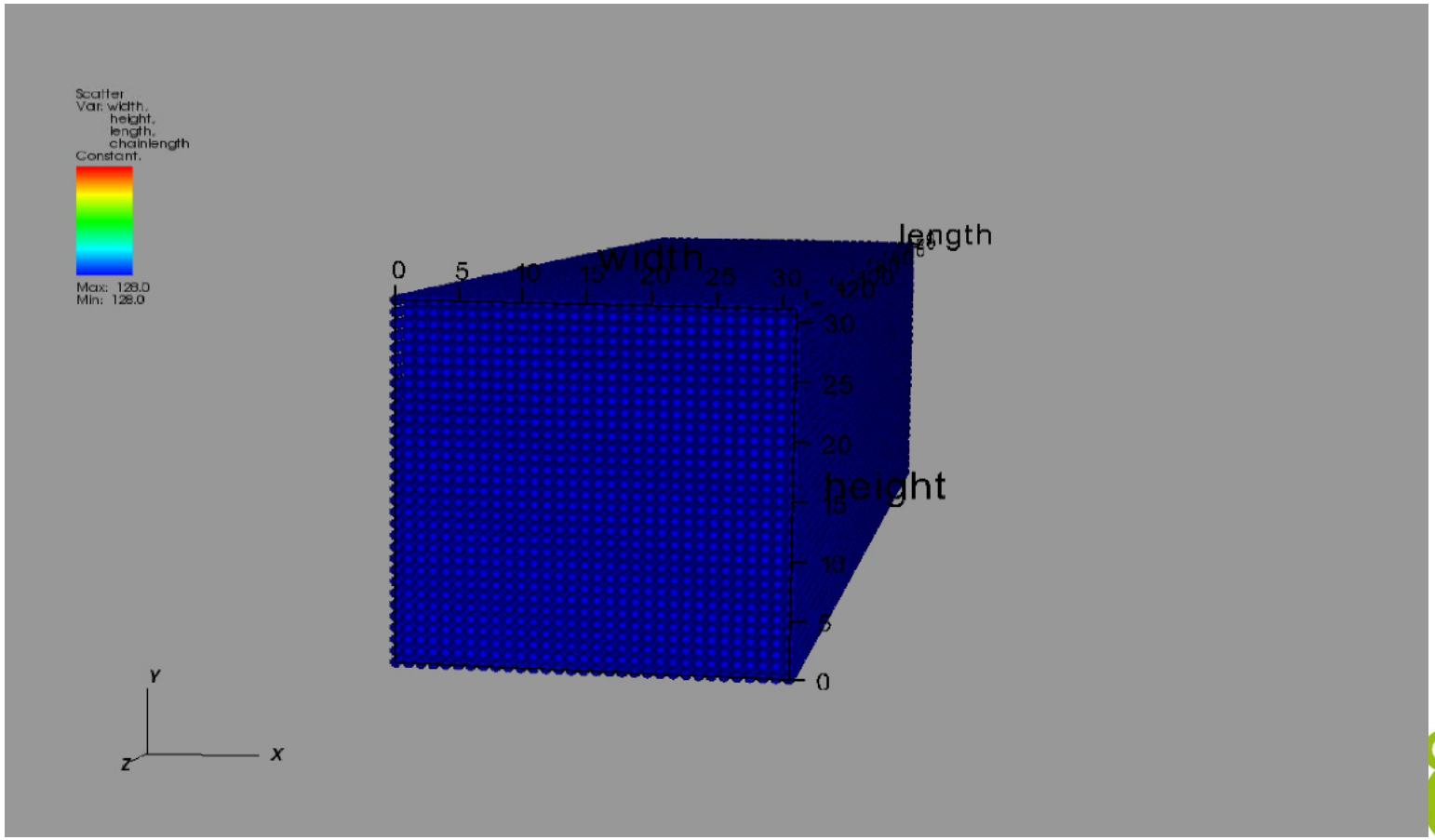


initial distribution



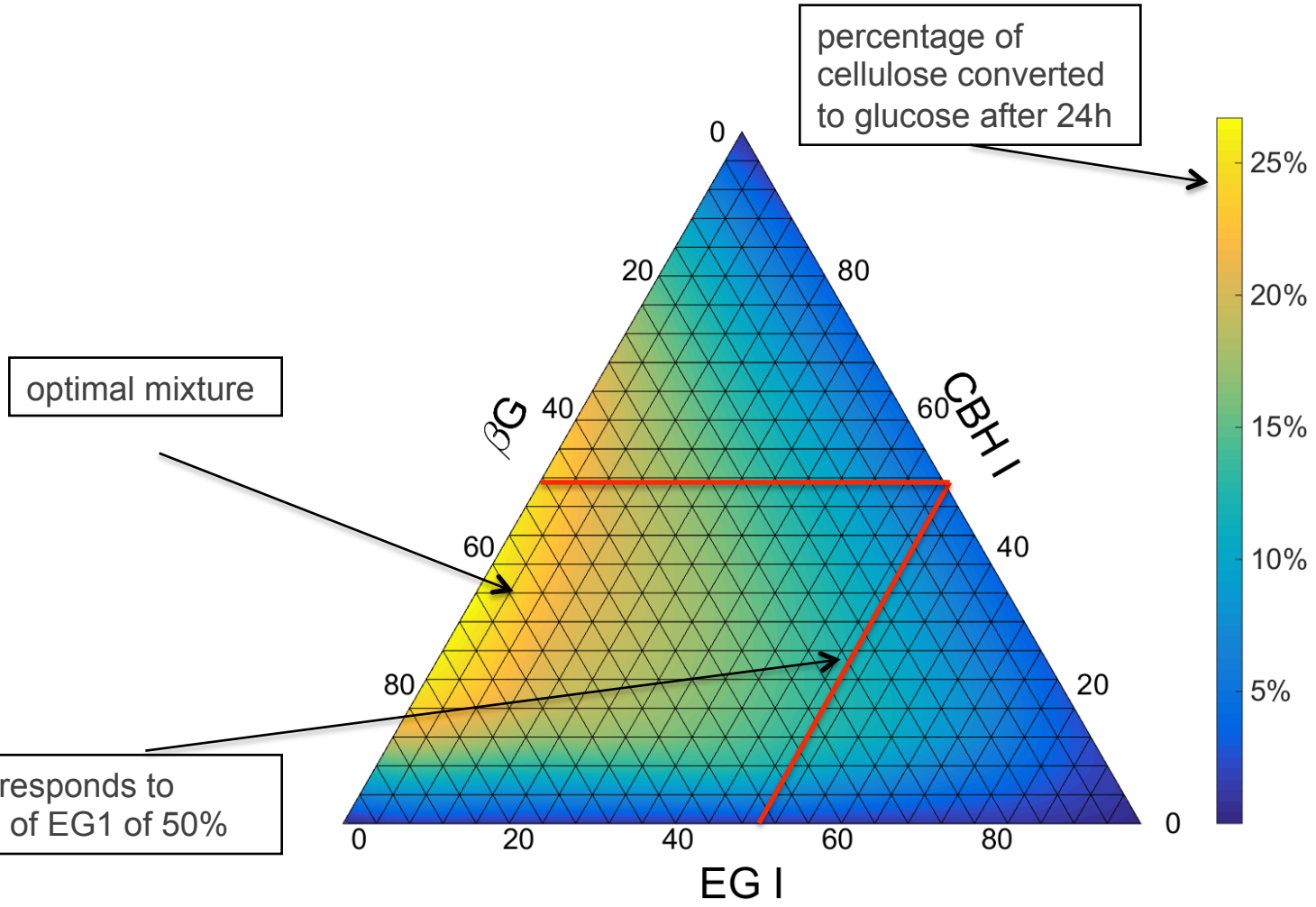
distribution after ~15h

WP6: Substrate degradation



WP6: Glucose production



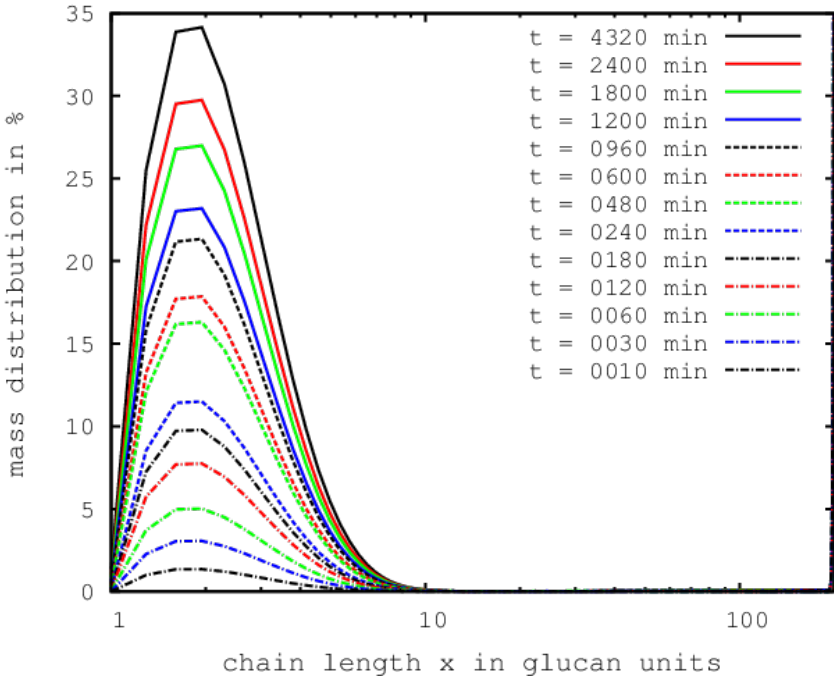


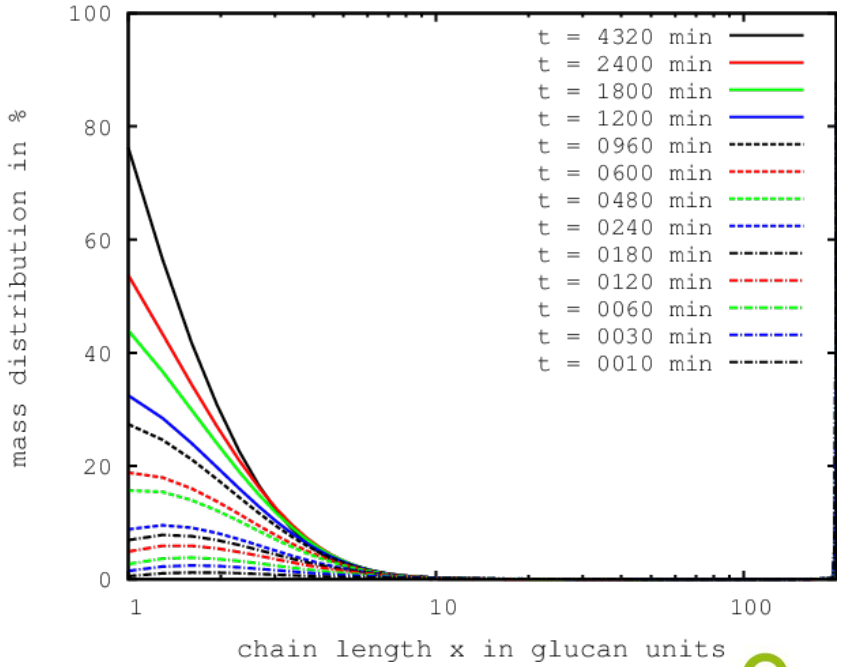
WP6: Polymer Mass Distⁿ by Time

CBH I only

Total enzyme mass: 20mg/g

[EG I] : [CBH I] : [βG] = 1: 1 : 1





Uniform initial chain length distribution: x = 200

Summary

- *What was proposed*

To develop a technology pipeline applying novel rapid combinatorial genetic methods supported by bioinformatics, expression tuning and efficient screening of enzyme / accessory protein cocktails.

- *What was achieved*

Development of a technology pipeline applying novel rapid combinatorial genetic methods supported by bioinformatics, expression tuning and efficient screening of enzyme / accessory protein cocktails.

Summary



- *Delayed start means 6 months to run*
  - Implement expression control techs for cellulases
  - Develop model to incorporate cell consumption
  - Use modelling data into inform expression levels
  - Implement systems in *Sc*

- *A well-functioning interdisciplinary team*
  - Royal Society Newton Award
    - Partnering with University of São Paulo
      - Functional metagenomics
    - Baggase as feedstock

Newton Fund

General Evaluation



- *Benefits of international collaboration*
- *Ouputs:*
  - *Two peer-reviewed publications*
  - *A number in preparation*
- *Effective interaction of researchers*
  - *Meetings, visits and virtual conferencing*
- *Adoption of technology by end-users*
  - *Early stage, traction via Ingenza customer base*

Contact details



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